Factors Affecting the Concentration of Sphingomyelin in Bovine Milk¹

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ABSTRACT

Sphingomyelin is a phospholipid located in the outer leaflet of the plasma membrane of most cells and is a component of the milk fat globule membrane. Sphingomyelin and its digestion products participate in several antiproliferative pathways that may suppress oncogenesis. Although milk and dairy products are important sources of sphingomyelin in the human diet, little is known about factors that influence sphingomyelin concentrations in milk fat or whether concentrations can be modified via the nutrition of cows. Sphingomyelin concentrations were determined in milk from Holstein and Jersey cows matched for parity and stage of lactation. Sphingomyelin was more concentrated in milk fat from Holstein cows than in milk fat from Jersey cows (1,044 vs. 839 $\mu\text{g/g}$ of fat). Concentrations in whole milk did not differ because of greater milk fat content for milk from Jerseys. Differences between breeds may be related to the greater fat globule size in milk from Jerseys. Sphingomyelin content in whole milk increased with increasing days in milk because of associated increases in milk fat content. Regardless of breed, primiparous cows had greater amounts of stearic acid and less palmitic acid in sphingomyelin than did older cows. The sphingomyelin concentration in milk fat of cows in a commercial Jersey herd was lower for cows in their fourth or greater parity. Sphingomyelin content in whole milk was greater for cows in late lactation because of greater milk fat content. Feed restriction of multiparous Holstein cows to 37% of ad libitum dry matter intake increased milk fat content but did not affect milk sphingomyelin content or milk fat globule size. Supplementation of the diet with 4% soybean oil

did not affect milk composition, sphingomyelin content, or milk fat globule size. Milk was sampled seasonally from 7 herds throughout Illinois during a 2-yr period. Sphingomyelin concentration in milk fat was greatest during summer and least during winter, but whole milk concentrations did not vary across seasons. We conclude that 1) sphingomyelin content of milk fat is greater in milk from Holsteins than that from Jerseys, 2) sphingomyelin content in whole milk increases with stage of lactation, and 3) sphingomyelin content of milk fat is greater during summer. However, efforts to produce milk with a greater sphingomyelin content through altering management and nutrition are unlikely to be successful.

Key words: sphingolipid, bovine milk fat globule membrane, milk fat

INTRODUCTION

Milk fat contains several compounds that are potentially beneficial for human health, including butyric acid, conjugated linoleic acid, ether lipids, and sphingomyelin (Parodi, 1997; Spitsberg, 2005). Sphingomyelin has been shown to be anticarcinogenic, particularly for colon cancer (Schmelz and Merrill, 1998). Milk fat is a particularly rich and convenient source of exogenous sphingomyelin in the human diet (Malmsten et al., 1994; Parodi, 1997). Sphingomyelin is a phospholipid found primarily in the outer leaflet of the plasma membrane of most mammalian cells (Schmelz et al., 1994; Merrill et al., 1997). When droplets of triacylglycerols are secreted from mammary cells, the droplets are enveloped by plasma membrane, thereby incorporating sphingomyelin and minor amounts of other sphingolipids into the milk fat globule membrane (Timmen and Patton, 1988; Spitsberg, 2005).

Phospholipids, in general, constitute a small percentage (<1%) of the total lipids in milk. Sphingomyelin contributes approximately one-quarter to one-third of the phospholipid portion (Bitman and Wood, 1990; Malmsten et al., 1994; Parodi, 1997), although more recent reports have indicated that sphingomyelin represents only 18 to 20% of the total phospholipids in milk (Avalli and Contarini, 2005; Rombaut et al., 2005). Quantitatively, therefore, the sphingomyelin concentration of milk is in the range of 4 to 12 mg/dL of milk (Bitman

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and Wood, 1990). Most of the sphingomyelin and other sphingolipids in milk originate in the milk fat globule membrane, and milk fat remains a major site of sphingomyelin concentration (Spitsberg, 2005). With homogenization and further processing of whole milk, however, the fat globule membrane is disrupted and some of the phospholipids, including sphingomyelin, move to the aqueous phase. Extensive processing therefore results in considerable enrichment of the whey or non-fat fractions with sphingomyelin (Rombaut et al., 2006). Because processing does not negatively influence milk concentrations of sphingomyelin, human consumption of whole and processed milk contributes greatly to the overall consumption of sphingolipids, which constitute from 0.01 to 0.02% of the diet (Vesper et al., 1999).

Once ingested, sphingomyelin is digested by alkaline sphingomyelinase throughout the length of the small intestine and colon, and its digestion products, ceramide and sphingosine, are absorbed by intestinal cells (Schmelz et al., 1994; Nilsson et al., 2003). Sphingomyelinase is inhibited by the presence of glycerides, fatty acids, and phospholipids, with the result that most sphingomyelin digestion and absorption of digestion products likely occurs in the distal small intestine and colon (Liu et al., 2002). Ceramide and sphingosine serve as second messengers in cell-signaling pathways that may modulate oncogenesis (Duan, 1998; Schmelz and Merrill, 1998; Schmelz, 2003). Sphingomyelin, in amounts similar to those in dairy products, has been shown to inhibit early colon carcinogenesis in mice and to decrease the number of malignant tumors in mice (Merrill et al., 1997; Vesper et al., 1999; Schmelz et al., 2000). Mice that were fed sphingomyelin and treated with 1,2-dimethylhydrazine to induce colon tumors had fewer aberrant colonic crypts, which are early markers of carcinogenesis, and decreased adenocarcinomas (Merrill et al., 1997).

Because sphingomyelin and other sphingolipids may help prevent or decrease the severity of colon cancer, it may benefit consumers to increase their consumption of sphingomyelin. Increasing the concentrations of sphingomyelin in milk and dairy products may therefore be of benefit to consumers. Consuming diets rich in dairy products may provide concentrations of sphingomyelin sufficient to prevent colon carcinogenesis and tumorigenesis (Schmelz et al., 2000).

Little is known about natural variations in the milk sphingomyelin content and the ability to manipulate sphingomyelin concentrations in milk and milk fat. Our objective was therefore to examine breed, physiological, dietary, and environmental factors that may affect sphingomyelin concentrations in milk and milk fat. More specifically, we sought to determine the effects of breed, stage of lactation, parity, and season on sphingo-

myelin concentrations in milk and milk fat. Furthermore, we explored the potential to modify sphingomyelin concentrations by altering the nutrient intake of cows, via either restriction of intake or soybean oil supplementation to the diet. Understanding natural variations in the sphingomyelin content and determining the ability to manipulate sphingomyelin concentrations in milk may lead to future increases in its content in dairy products for human consumption.

MATERIALS AND METHODS

All experimental procedures involving cows were approved by the University of Illinois Institutional Animal Care and Use Committee.

Experimental Design and Sampling

Sphingomyelin in Jersey vs. Holstein Milk (Experiment 1). Whole milk samples (approximately 500 mL) from 23 Holstein and 23 Jersey cows from the University of Illinois dairy herd were obtained on 2 consecutive days from both the morning and evening milkings in 2 consecutive years (4 milkings total each year). Cows sampled in year 1 were not necessarily the same as those sampled in year 2. For each year, cows were matched between breeds based on parity (1, 2, 3, or ≥ 4) and stage of lactation (<100 DIM = early lactation; 100 to 180 DIM = mid lactation; >200 DIM = late lactation). Milk weights were recorded for each milking. Milk samples were composited each day in proportion to milk yield. Milk sample composites were analyzed for contents of fat using midinfrared techniques (AOAC, 1995) at a commercial laboratory (Dairy Lab Services, Dubuque, IA). Milk fat was prepared and stored at -20°C until analysis for sphingomyelin content and fatty acids attached to sphingomyelin using methods described in the following sections.

Sphingomyelin in Milk of a Commercial Jersey Herd (Experiment 2). Milk samples (approximately 500 mL) were collected from cows in a commercial Jersey herd in southern Illinois. Samples were obtained from 32 cows divided evenly among parity number (parity 1 = 8; parity 2 = 8; parity 3 = 8; parity ≥ 4 = 8). Half of the cows in each parity group were in early lactation (<100 DIM) and half were in late lactation (>200 DIM). Morning and evening milk samples were collected and composited in proportion to milk yield. Composite samples were analyzed for contents of fat as described for experiment 1. Milk fat was collected and stored at -20°C until analyzed for sphingomyelin content.

Feed Restriction and Milk Sphingomyelin (Experiment 3). Six Holstein cows were housed in individual tie stalls and fed a diet balanced to meet or exceed

recommendations for all nutrients (NRC, 2001). The diet was based on corn silage, alfalfa silage, ground corn grain, soybean hulls, and soybean meal with appropriate mineral and vitamin supplementation. The diet was fed as a TMR for ad libitum DMI or progressively restricted to 64, 50, and 37% of initial ad libitum DMI for 1-wk periods (Beaulieu et al., 2001). During the first 3 wk, all cows were fed ad libitum; 3 cows were then restricted progressively for the next 3 wk while the other 3 cows remained on ad libitum intake. Milk samples were collected once weekly from morning and evening milkings and were composited in proportion to milk yield. Milk samples were analyzed for milk fat particle size distribution (Smith et al., 1995) and composition as described for experiment 1. Milk fat was collected from the remaining composite sample and stored (-20°C) until analysis for sphingomyelin content.

Soybean Oil Supplementation and Milk Sphingomyelin (Experiment 4). Six Holstein cows were housed in tie stalls and fed either the same control diet as described for experiment 4 or the control diet plus 4% soybean oil (Beaulieu et al., 2001). During the first week, all cows were fed the control diet; in wk 2, 3 cows were fed the control diet plus 3% soybean oil (DM basis), and 3 cows remained on the control diet. During the next 3 wk, the experimental group received the control diet plus 4% soybean oil (DM basis). Once weekly, milk samples were collected from morning and evening milkings and samples were composited based on the proportion of milk yield. Milk samples were analyzed for milk fat particle size distribution (Smith et al., 1995) and composition as described for experiment 1. Milk fat was collected from the remaining milk sample and stored at -20°C until analyzed for sphingomyelin content.

Season and Sphingomyelin in Milk from Illinois Dairy Herds (Experiment 5). Bulk tank milk samples (approximately 500 mL) were collected on a quarterly basis from 7 dairy herds throughout Illinois over a 2-yr period. Three Holstein herds (northern, southern, and central regions), 2 Jersey herds (southern and central regions), 1 Guernsey herd (central region), and 1 Brown Swiss herd (northern region) were sampled. Milk fat content was obtained from DHIA analyses. Milk fat was collected and stored at -20°C until analyzed for sphingomyelin content.

Sphingomyelin Assay

Whole milk collected from individual cows in all experiments was centrifuged at $10,000 \times g$ and 4°C for 10 min. Milk fat was then removed from the top of the tube, placed in scintillation vials, flushed with nitrogen gas, and frozen (-20°C) until analysis. Preliminary experiments demonstrated that $<10\%$ of the total milk

sphingomyelin was found in the nonfat fraction. Therefore, absolute quantities of milk sphingomyelin are underestimated. Because our aim was to compare milk samples from different physiological situations, we chose to analyze only the milk fat fraction, in which sphingomyelin was enriched.

Before analysis, fatty acids and other lipids were extracted from the milk fat using a method modified from Folch et al. (Folch et al., 1957; Bowyer and King, 1977; Christie, 1982). One gram of milk fat was weighed into a screw-topped tube (25×150 mm Pyrex). Twenty milliliters of chloroform:methanol (2:1) was added to the tube, which was then vortexed for 30 s. Four milliliters of NaCl (0.9%) was added, and the tube was inverted to mix the contents and then centrifuged at $1,500 \times g$ for 10 min. The upper aqueous layer was removed and discarded and the bottom organic layer was dried under nitrogen gas. After the organic extract was dry, 1 mL of chloroform:methanol (2:1) and 2 mL of 0.9% NaCl were added, and the tube was vortexed and then centrifuged at $1,500 \times g$ for 5 min. Again, the upper aqueous layer was discarded and the lower layer was saved and dried under nitrogen. One milliliter of chloroform was added to the dry lower layer, and the tube was vortexed. Thin-layer chromatography (TLC) was then used to separate sphingomyelin from the other lipids (Zeisel et al., 1986).

The chloroform extract of milk fat (20 μL per lane) was spotted onto TLC plates (LK6D Silica Gel 60A, 250 μm thick; Whatman Inc., Clifton, NJ). Each sample was spotted onto 6 lanes. Samples were run in triplicate with 2 lanes per tube (40 μL per tube). Multiple lanes were used to prevent sample overload in the lanes. Three standard mixtures (25 μL each) were spotted onto each plate, including a polar lipid mixture (Nu-Chek 18-5A; Nu-Chek Prep, Elysian, MN) and 2 other standards (Matreya, Pleasant Gap, PA; sphingomyelin, sphingolipid mix, brain extract, serum lipid mix, or phospholipid mix) to determine where sphingomyelin separated on the plate. All standards and samples were spotted using a 25- μL Teflon-lined Hamilton syringe. Dual TLC was used to separate the lipids. The first TLC chamber contained 90 mL of acetone to soak the filter paper in the chamber and 10 mL of acetone in the tray; the plate was placed in the tray and the solvent was allowed to run to the top of the plate to remove the triglycerides and FFA (Zeisel et al., 1986). Once the plate was dry, it was placed in the second chamber containing 90 mL of solvent mix (750 mL of chloroform, 250 mL of methanol, and 30 mL of distilled water). Solvent was allowed to run to 0.45 cm from the top of each plate. The plate was then removed and allowed to air dry. The plate was stained with iodine to identify all bands separated (Zeisel et al., 1986). After the iodine

volatilized from the plate, it was sprayed with molybdenum blue spray reagent (M3389; Sigma Chemical Co., St. Louis, MO) to identify the phospholipid bands. Sphingomyelin bands were identified based on comparison with standard separation, and the 2 bands from the 2 lanes representing sphingomyelin from 40 μ L of milk fat were scraped into a corresponding screw-topped tube (16 $\times$ 150 mm Pyrex). Thus, there were 3 tubes for each sample.

A standard curve was prepared to quantify sphingomyelin content of samples based on phosphorus content. The standard stock solution was prepared by dissolving sphingomyelin from buttermilk (Matreya 1239; Matreya) in 250 mL of chloroform to a concentration of 100 μ g/mL. The stock solution was then diluted with chloroform to obtain standard concentrations of 90, 80, 70, 60, 50, 40, 30, 20, 10, and 0 μ g/mL. One milliliter of each standard was placed in a tube (16 $\times$ 150 mm Pyrex) and dried under nitrogen before the sphingomyelin assay; each standard was prepared in duplicate. Standards then were treated similarly to other samples.

Recovery samples also were run with each assay performed. Composite milk fat sample standards from our laboratory were treated in the same manner as the sphingomyelin milk fat samples described above. Two milk fat standards were extracted for each assay. Six lanes of each sample (in triplicate) were spotted. In addition, 40 μ L of extracted milk fat standard was dried under nitrogen and then spiked with 1 mL of sphingomyelin standard of approximately 60 μ g/mL concentration (the exact concentration was known each time). Spiking also was done in triplicate. Spiked samples were dried under nitrogen and 40 μ L of chloroform was added to each dried tube to correspond to the amount of unspiked milk fat spotted onto the TLC plates. Spiked samples were spotted and treated like other samples. One milliliter of spike solution was dried under nitrogen (triplicate for each sample) and then used directly in the assay. The recovery from the assay was calculated as (spiked milk fat sphingomyelin concentration – unspiked milk fat sphingomyelin concentration)/spike sphingomyelin concentration.

Recovery averaged 55% for all assays, and final concentrations of samples were corrected for this recovery. The same milk fat standards were used in all assays to allow comparison of concentrations among assays to detect possible errors in individual assay runs.

Sphingomyelin was quantified by measuring the amount of phosphorus in sample spots using a modification of the method of Eng and Noble (1968). All tube tops were wrapped with Teflon tape and 0.9 mL of 70% perchloric acid was added. Tubes were placed in a sand bath (190°C) for 1 h. After 1 h, tubes were removed from the sand bath and allowed to cool. When tubes

were cool, 7 mL of distilled water, 0.5 mL of 2.5% ammonium molybdate (Fisher Scientific, Pittsburgh, PA), and 0.2 mL of Fiske and Subbarow reducer (aminonaphthosulfonic acid reagent, 661-8; Sigma Chemical Co.) were added to each tube. Tubes were vortexed and placed in a boiling water bath for 7 min. After cooling, tubes were centrifuged at $1,500 \times g$ for 10 min to precipitate the silica gel from the TLC plates. An aliquot of the supernatant from each tube was placed in a microcuvette (A-130 disposable cells; Spectrocell, Oreland, PA) and absorbance was determined in a spectrophotometer at a wavelength of 820 nm (Eng and Noble, 1968; Zeisel et al., 1986).

Analysis of the Fatty Acid Composition of Sphingomyelin

Milk fat was extracted and TLC was performed as described to separate sphingomyelin. Each sample was analyzed in duplicate; thus, only 4 lanes were spotted for TLC. After staining, 2 bands of sphingomyelin from 2 lanes were scraped into each tube, and acid-catalyzed methylation (Sukhija and Palmquist, 1988) was used to methylate the fatty acids. Four milliliters of benzene was added to the scrapings in each tube, the tubes were vortexed, and 3 mL of 10% methanoic HCl (20 mL of acetyl chloride in 200 mL of anhydrous methanol) was added. Teflon tape was wrapped around the top of each tube, and the tubes were capped tightly and then vortexed. The tubes were incubated at 70°C for 2 h and were carefully mixed once during incubation. After cooling samples on ice to room temperature, 5 mL of 6% K_2CO_3 (60 g of K_2CO_3 in 1 L of distilled water) was added to each tube. The tubes were shaken vigorously and then centrifuged for 5 min at $1,500 \times g$. The upper organic layer was removed, transferred to cold chromatography vials, and dried under nitrogen gas. Benzene (100 μ L) was added to each vial. Methylated samples were analyzed on a 100-m SP-2560 column (Supelco, Bellefonte, PA) in a gas chromatograph (GC-17A; Shimadzu Corporation, Kyoto, Japan) equipped with an autosampler and a flame-ionization detector. Helium was the carrier gas (294 kPa for 10 min, and 354 kPa for 30 min), and column temperatures were held at 120°C for 4 min and then held at 220°C for 20 min, with a total run time of 44 min. Peaks were identified based on a comparison of retention times with authentic standards (Matreya; Nu-Chek Prep), and identification is therefore presumptive.

Statistical Analysis

All analyses in each experiment were performed by using SAS (version 8e, 1999; SAS Institute Inc., Cary, NC).

Experiment 1. Data for concentrations of sphingomyelin in whole milk and in milk fat, and for milk fat content were analyzed using the MIXED procedure in SAS. The model included the effects of stage, breed, parity, and day of sampling, and the interactions of breed with parity, day of sampling, and both parity and day of sampling, as well as the interaction of parity with day of sampling. Block and cow were defined as random effects. Day of sampling for each cow was specified as a repeated measure. Several covariance structures were examined and the one resulting in the smallest Akaike's information criterion (autoregressive 1) was chosen as the most appropriate (Littell et al., 1996). The analysis of area percentages of fatty acids attached to sphingomyelin was performed by using the MIXED procedure in SAS. Breed, parity, and the interaction of breed with parity were included in the model. Least squares means and standard errors are reported. Parity effects were separated using the predicted difference (PDIF) option in SAS.

Experiment 2. The MIXED procedure was used to analyze data. Cow was defined as a random effect. The effects of parity, stage of lactation, and the interaction of these parameters on sphingomyelin in whole milk and milk fat and on milk fat percentage were determined. Least squares means and standard errors are reported, with significance at $P < 0.05$. Parity effects were separated using the PDIF option in SAS.

Experiments 3 and 4. Results for restricted intake and soybean oil supplementation were analyzed in the same manner (Beaulieu et al., 2001). The relationships among individual milk fatty acids, sphingomyelin in milk and milk fat, the volume mean diameter of milk fat globules (VMD), the D(90) value (the diameter of milk fat globules below which 90% of milk fat is contained), and milk fat percentage were determined using the CORR procedure in SAS. Violations of model assumptions existed; therefore, the Spearman's rho function was used to calculate correlations.

The MIXED procedure in SAS was used to analyze treatment effects. The model included the effects of treatment (either degree of feed restriction or amount of soybean oil fed), cow nested within treatment, week in trial, and the interaction between treatment and week in trial. In this design, week and level of treatment factors (feed restriction or soybean oil supplementation) were confounded; therefore, the interaction of treatment and week was the parameter of interest. Least squares means and standard errors are reported.

Experiment 5. The effect of season on sphingomyelin concentrations in bulk tank milk from farms in various regions of Illinois was tested using the MIXED procedure of SAS. Season was analyzed as a repeated measurement, with farm nested within year as the subject.

Table 1. Sphingomyelin concentrations in milk and milk fat from Holstein and Jersey cows (experiment 1)

Component	Breed		SEM
	Holstein	Jersey	
Milk fat, %	3.49	4.31*	0.17
Sphingomyelin, $\mu\text{g/g}$ of milk fat	1,044	839*	124
Sphingomyelin, $\mu\text{g/g}$ of milk	35.0	34.9	2.6

*Different ($P < 0.01$) from Holstein.

Several covariance structures were examined, and the one that resulted in the smallest Akaike's information criterion (autoregressive 1) was chosen as the most appropriate (Littell et al., 1996). Year was designated a random effect. When the effect of season was significant ($P < 0.05$), means were separated by using the PDIF option. Correlation analysis was conducted using the CORR procedure in SAS.

RESULTS AND DISCUSSION

Sphingomyelin in Jersey vs. Holstein Milk (Experiment 1)

Using a sampling of Holstein and Jersey cows matched for parity and stage of lactation, we explored the effects of breed, parity, and stage of lactation on concentrations of sphingomyelin in milk and milk fat. Jersey milk had a greater ($P < 0.001$) percentage of milk fat than did Holstein milk (4.31 vs. 3.49%; Table 1). Holstein milk fat contained greater ($P < 0.003$) concentrations of sphingomyelin (1,044 $\mu\text{g/g}$ of milk fat) than did Jersey milk fat (839 $\mu\text{g/g}$ of milk fat; Table 1). Whole milk concentrations of sphingomyelin did not differ ($P = 0.73$) between breeds (35.0 vs. 34.9; Table 1). Parity number did not affect the sphingomyelin concentrations in milk fat ($P = 0.45$) or whole milk ($P = 0.65$). Stage (i.e., DIM) did not affect sphingomyelin concentrations in milk fat ($P = 0.95$). A trend ($P = 0.07$) was detected for increased sphingomyelin concentrations in whole milk with increasing DIM, which followed the pattern for change in milk fat percentage ($P < 0.01$) with increasing DIM (data not shown).

Mean concentrations of sphingomyelin in milk fat were similar to values reported by others. For example, Avalli and Contarini (2005) reported that sphingomyelin concentration in milk fat from whole milk was 736 $\mu\text{g/g}$ of fat, and Rombaut et al. (2005) found concentrations of 1,269 $\mu\text{g/g}$ of fat. Our values for the concentration of sphingomyelin in whole milk were at the low range of concentrations (39 to 119 $\mu\text{g/g}$ of milk) reported by Bitman and Wood (1990).

The well-known fact that milk fat globule size is smaller for Holsteins than for Jerseys (Campbell, 1932)

may be responsible for the greater sphingomyelin concentrations in Holstein milk fat. At least part of the breed effect in milk fat globule size is related to milk fat yield, because fat globule size increases with greater milk fat production (Timmen and Patton, 1988; Wiking et al., 2004). The smaller size of the milk fat globule for Holsteins means a greater milk fat globule membrane surface area is present relative to the core lipid volume. In consequence, because of increased membrane surface, a greater concentration of sphingomyelin is found in Holstein milk fat. The greater milk fat content of Jersey milk negated the greater sphingomyelin content of Holstein milk fat, which resulted in similar sphingomyelin concentrations in whole milk between breeds. In other words, the larger milk fat globule size for Jerseys leads to the presence of less sphingomyelin-containing membrane per unit of milk fat, but the presence of more milk fat compensates for the larger globule size when comparing whole milk sphingomyelin concentrations. Consumption of fluid milk from one breed vs. the other likely would not be of any benefit in increasing sphingomyelin concentrations for prevention of colon cancer. However, for products that contain high percentages of fat, using Holstein milk, with its greater sphingomyelin concentrations in milk fat, would provide slightly larger amounts of sphingomyelin, possibly increasing protective effects.

The trend for increased whole milk sphingomyelin with increasing DIM also is explained by increases in the milk fat content with increasing DIM. Increased milk fat content in later lactation means that more milk fat globules are present; consequently, more sphingomyelin-containing membrane would be present. Thus, whole milk sphingomyelin concentrations tended to increase.

Breed did not affect the profile of fatty acids associated with sphingomyelin (data not shown). The interaction of breed and parity also was not significant, but parity affected some fatty acids associated with sphingomyelin (Table 2). In cows in second or greater parity, 16:0 was the most abundant fatty acid in sphingomyelin, followed by 18:0, 18:1n-9cis, 22:0, and 14:0. Substantial amounts of 23:0 and 24:0 also were detected. In first-parity cows, however, the most prevalent fatty acid was 18:0, followed by 16:0 and 22:0. Cows in their second or greater parity had greater contents of 14:0 and 16:0 in sphingomyelin than did first-parity cows. The greater amount of longer chain fatty acids (≥ 18 C) attached to sphingomyelin in first-parity cows indicates that they derived more of the fatty acids from blood lipids than from de novo synthesis in the mammary gland. Our results indicate either that more of these fatty acids were required for continuing growth and development of the mammary gland in first-parity

cows, and therefore more were removed from the blood, or that the supply of these fatty acids to the mammary gland was greater in first-parity cows. The fatty acids in sphingomyelin did not necessarily reflect the overall milk fatty acid composition (data not shown), which would tend to support the first possibility.

Although statistical differences in the fatty acids attached to sphingomyelin were noted among parities, whether these small differences may be biologically significant for the physiology of the cow or to human health is not known. In general, the profile of fatty acids was similar to that in a previous report (Bitman and Wood, 1990). The effects of parity and stage of lactation on sphingomyelin concentrations were explored in more detail in experiment 2.

Sphingomyelin in Milk from a Commercial Jersey Herd (Experiment 2)

The effects of parity and stage of lactation on sphingomyelin concentrations were further examined by sampling only Jerseys of various parities and stages of lactation. Sphingomyelin concentrations in milk fat were influenced by parity ($P < 0.05$; Table 3). Sphingomyelin concentrations in milk fat from cows in their first (769 $\mu\text{g/g}$ of fat), second (794 $\mu\text{g/g}$ of fat), and third (802 $\mu\text{g/g}$ of fat) parity were greater ($P < 0.05$) than the concentrations in milk fat from cows in their fourth or greater parity (652 $\mu\text{g/g}$ of fat; Table 3). Overall, whole milk concentrations of sphingomyelin were not affected by parity number ($P = 0.10$), but a decrease ($P < 0.05$) was noted between the third and fourth parities (37.0 vs. 26.4 $\mu\text{g/g}$ of milk; Table 3). Stage of lactation affected whole milk concentrations of sphingomyelin ($P < 0.001$), but not concentrations of sphingomyelin in milk fat ($P = 0.74$). Cows in late lactation (>200 DIM) had greater ($P < 0.05$) concentrations (38.2 $\mu\text{g/g}$ of milk) in milk than did cows in early lactation (<100 DIM; 24.7 $\mu\text{g/g}$ of milk). No interaction between parity and stage of lactation was noted for either milk fat or whole milk sphingomyelin concentrations. Milk fat percentage was not affected by parity ($P = 0.57$), but cows in late lactation had a greater milk fat content ($P < 0.001$) than cows in early lactation (4.46 vs. 3.00%).

The effect of parity on sphingomyelin concentration in milk fat is difficult to explain because little research is available on changes in milk fat globule size with increasing lactation number. Previous research indicated that milk fat globule size decreases as lactation number increases (Campbell, 1932), which could lead to increased sphingomyelin concentrations in milk fat with increasing parity. However, the results from our study indicate that the milk fat globule size might actually have increased in cows in their fourth or greater

Table 2. Effect of parity on the profile of fatty acids associated with sphingomyelin in milk fat (experiment 1)

Fatty acid	Lactation number			
	1	2	3	4
	(g/100 g)			
14:0	3.22 ^b ± 0.82	7.60 ^a ± 0.96	6.97 ^a ± 0.96	7.77 ^a ± 1.15
16:0	21.49 ^b ± 2.04	33.47 ^a ± 2.38	30.79 ^a ± 2.38	33.67 ^a ± 2.85
16:1n-7 <i>cis</i>	4.24 ± 1.08	1.75 ± 1.23	4.03 ± 1.26	3.27 ± 1.51
18:0	29.82 ^a ± 1.24	25.76 ^b ± 1.44	26.22 ^b ± 1.44	27.97 ^{ab} ± 1.73
18:1n-9 <i>cis</i>	7.55 ^c ± 1.30	14.78 ^a ± 1.51	11.83 ^{ab} ± 1.51	9.78 ^{bc} ± 1.81
18:1n-7 <i>trans</i>	3.79 ^a ± 0.59	2.01 ^{ab} ± 0.69	1.16 ^b ± 0.69	1.85 ^{ab} ± 0.82
18:2n-6	0.23 ^b ± 0.08	0.45 ^a ± 0.13	0.38 ^{ab} ± 0.13	0.49 ^b ± 0.22
22:0	14.53 ^a ± 1.53	6.48 ^b ± 1.78	10.29 ^{ab} ± 1.78	7.34 ^b ± 2.13
23:0	8.15 ^a ± 0.85	3.30 ^b ± 0.98	3.16 ^b ± 0.98	3.97 ^b ± 1.18
24:0	5.98 ^a ± 0.70	3.11 ^b ± 0.82	4.47 ^b ± 0.82	3.50 ^b ± 0.98
Other ¹	1.02 ± 0.32	1.28 ± 0.37	0.71 ± 0.37	0.41 ± 0.40

^{a-c}Means within a row having different superscript letters differ ($P < 0.05$).

¹Other fatty acids identified in some, but not all, samples included 14:1, 18:1 *trans* isomers other than n-7 *trans*, 18:3n-3, 20:0, 21:0, and 24:1.

parity, although this was not measured directly. Although milk fat percentage was not affected by parity, older cows may have produced more milk fat (not determined), which could lead to a larger mean globule size (Wiking et al., 2004). The increased fat globule size would cause the decrease in milk fat sphingomyelin seen here; however, because globule size data were not collected, this possibility remains speculative. Alternatively, the smaller sphingomyelin concentrations in milk fat in cows of fourth or greater parity may have been caused by other factors. For example, the milk fat globule membrane may become thinner or there may be less sphingomyelin in the milk fat globule membrane with increased parity. Either of these circumstances would decrease the sphingomyelin concentrations in milk fat and, consequently, in whole milk.

The increase in whole milk concentrations with increased stage of lactation may be caused by several factors. Milk fat content increased with advancing stages of lactation, and this likely had the greatest influence on sphingomyelin content in whole milk. Increased amounts of milk fat would lead to greater concentrations of sphingomyelin in whole milk. Previous studies have reported an increase in the amount of milk fat globule membrane in milk with increased stages of lactation, indicating a decrease in globule size (Hofi et

al., 1977). Furthermore, an increase in the amount of sphingomyelin in the membrane with increased stages of lactation was reported previously (Hofi et al., 1977). The decrease in globule size and increase in sphingomyelin in the membrane also could contribute to the increased sphingomyelin concentrations in whole milk with increased stage of lactation. These effects likely are minor contributors here, however, because concentrations of sphingomyelin in milk fat were not affected by stage of lactation. Regardless, isolating late-lactation cows from the rest of the herd would be an impractical approach to obtain milk with an increased sphingomyelin content for potential colon cancer prevention.

Feed Restriction in Milk Sphingomyelin (Experiment 3)

Impact of the overall plane of nutrition on sphingomyelin concentrations was measured because feed restriction may alter milk fat percentages, primarily by decreasing milk production. Milk production decreased ($P < 0.01$) and milk fat percentage increased ($P < 0.01$) as the degree of feed restriction increased (Table 4). Sphingomyelin concentrations in whole milk were weakly correlated (Spearman's $\rho = 0.21$, $P < 0.05$) with the D(0.90) value of milk fat globules and with

Table 3. Effect of parity on sphingomyelin concentration in a commercial Jersey herd (experiment 2)

Component	Parity number			
	1	2	3	≥4
Sphingomyelin, µg/g of milk fat	769 ^a ± 44	794 ^a ± 49	801 ^a ± 56	651 ^b ± 63
Sphingomyelin, µg/g of milk	30.0 ^{ab} ± 3.3	32.4 ^{ab} ± 3.6	37.0 ^a ± 4.0	26.4 ^b ± 4.4

^{a,b}Means within a row having different superscript letters differ ($P < 0.05$).

Table 4. Effects of feed restriction on milk yield, milk composition, and sphingomyelin concentrations (experiment 3)¹

Item	Treatment		SEM
	Ad libitum	Restricted	
DMI, kg	22.6	8.4*	0.3
Milk yield, kg	28.7	17.6*	1.3
Milk fat, %	3.86	4.50*	0.14
Sphingomyelin, $\mu\text{g/g}$ of milk fat	1,134	1,203	84
Sphingomyelin, $\mu\text{g/g}$ of milk	44.0	54.1	3.5
Milk fat VMD, ² μm	3.14	3.01	0.046
Milk fat D(0.9), ³ μm	6.20	5.99	0.11

¹Restricted cows were progressively restricted from ad libitum DMI to 63, 50, and 37% of ad libitum intake for 1-wk periods. Data shown are from the final week of feed restriction (37% of previous ad libitum intake).

²Volume mean diameter of milk fat globules.

³Diameter of milk fat globules below which 90% of the volume of milk fat is contained.

*Interaction of treatment and week ($P < 0.01$); values shown for restricted cows differ from those fed ad libitum.

milk fat percentage (Spearman's $\rho = 0.31$, $P < 0.01$). Feed restriction did not affect sphingomyelin concentrations (Table 4) in milk fat, however, as indicated by the lack of interaction between treatment and week for sphingomyelin in either milk fat ($P = 0.57$) or whole milk ($P = 0.22$). Individual variation among cows accounted for the major differences in sphingomyelin concentrations in milk fat ($P < 0.03$), but not in whole milk ($P = 0.59$).

Neither volume mean diameter of the milk fat globules nor the D(0.90) value was changed by feed restriction, indicating that the milk fat globule size was unaffected. A consistent globule size may explain why concentrations of sphingomyelin in milk fat were not changed by feed restriction. The increase in fat percentage may not have been dramatic enough to alter whole milk concentrations of sphingomyelin.

Soybean Oil Supplementation and Milk Sphingomyelin (Experiment 4)

Feeding supplemental fats and oils changes milk fatty acid composition (Palmquist et al., 1993). The fatty acid composition of milk fat globule membrane is more difficult to change by dietary fat (Palmquist and Schanbacher, 1991); nevertheless, supplementation of fat to the diet might be expected to alter the amount of sphingomyelin in milk fat globule membranes, particularly if the fat globule size were affected. We chose to supplement soybean oil because it is a source of unsaturated long-chain fatty acids, the majority of which would be at least partially hydrogenated in the rumen. We reasoned that the combination of escape of some long-chain polyunsaturated fatty acids, the ruminal production of vari-

ous *trans*-unsaturated intermediates, and the possibility of decreased milk fat content as a result of feeding soybean oil (Lock and Bauman, 2004) might have greater potential to alter the milk fat globule size, structure, and sphingomyelin content.

In contrast to our hypothesis, however, supplementation of the diet with 4% soybean oil had no effect on sphingomyelin concentrations (Table 5) in milk fat or in whole milk, as indicated by the lack of interactions between treatment and week for either concentrations in milk ($P = 0.74$) or milk fat ($P = 0.74$). The effect of individual cow variation was not present as it was in the restricted feed intake study (experiment 3). Soybean oil supplementation did not affect milk yield, milk fat percentage, fat globule volume mean diameter, or fat globule D(0.90) value (Table 5). Although milk fat composition was altered as expected (data not shown), the lack of changes in milk fat content and fat globule size likely resulted in the unaltered sphingomyelin concentrations in milk fat between the control cows and those with soybean oil-supplemented diets.

These results indicate that it may be difficult to alter the concentrations of sphingomyelin in milk or milk fat by changing feeding strategies, such as by addition of fat. The ability to alter sphingomyelin concentrations in milk or milk fat by restricting intake (experiment 3) or by supplementation with soybean oil (experiment 4) was limited; therefore, it may be difficult to obtain milk with increased sphingomyelin concentrations to benefit human health by changing the diets of dairy cows.

Sphingomyelin in Milk from Illinois Dairy Herds Across Seasons (Experiment 5)

This study examined herd-level concentrations of sphingomyelin in bulk milk of farms throughout the

Table 5. Effects of supplementation of 4% soybean oil to the diet on milk yield, milk composition, and sphingomyelin concentrations (experiment 4)¹

Item	Treatment		SEM
	Control	Soybean oil	
DMI, kg	24.8	25.0	0.6
Milk yield, kg	30.6	29.4	1.1
Milk fat, %	3.66	3.45	0.26
Sphingomyelin, $\mu\text{g/g}$ of milk fat	1,050	1,254	180
Sphingomyelin, $\mu\text{g/g}$ of milk	34.0	36.7	6.8
Milk fat VMD, ² μm	2.73	2.70	0.13
Milk fat D(0.9), ³ μm	5.12	5.18	0.23

¹Cows were fed a diet containing 3% (DM basis) soybean oil during wk 1 and then 4% soybean oil during wk 2 to 4. Data shown are from the final week of supplementation. The interaction of treatment and week was not significant for any variable.

²Volume mean diameter of milk fat globules.

³Diameter of milk fat globules below which 90% of the volume of milk fat is contained.

Table 6. Least squares means and associated standard errors for the effect of season on sphingomyelin concentrations in milk fat and in whole milk sampled in bulk milk from 7 Illinois dairy herds (experiment 5)

Variable	Season			
	Fall	Winter	Spring	Summer
Sphingomyelin, $\mu\text{g/g}$ of milk fat	996 $\pm$ 57 ^{ab}	949 $\pm$ 49 ^b	970 $\pm$ 42 ^b	1,100 $\pm$ 44 ^a
Sphingomyelin, $\mu\text{g/g}$ of milk	41.2 $\pm$ 2.6	39.9 $\pm$ 2.2	40.2 $\pm$ 1.9	44.6 $\pm$ 2.0
Milk fat, %	4.15 $\pm$ 0.24	4.26 $\pm$ 0.18	4.20 $\pm$ 0.17	4.10 $\pm$ 0.18

^{a,b}Least squares means having different superscript letters differ ($P < 0.05$).

year. Herds varied in breed and geographic location in the state and were sampled repeatedly in different seasons. Sphingomyelin concentrations in milk fat were greatest during the summer and least during the winter; concentrations in the spring and fall were intermediate (Table 6). Sphingomyelin content in whole milk did not vary significantly among seasons (Table 6). Although the overall effect for season was not significant ($P = 0.13$) for milk fat content, the comparison of winter vs. summer ($P = 0.05$) revealed that the least milk fat was produced during the summer (Table 6). Across all seasons and farms, the sphingomyelin concentration in milk fat was negatively correlated ($r = -0.47$; $P < 0.05$) with milk fat content. Because milk fat droplet size increases with greater milk fat content, sphingomyelin content in milk fat might be expected to decrease, as discussed for experiment 2.

Variation of sphingomyelin concentration in milk fat and whole milk within and among herds was considerable. A pasture-fed herd in northern Illinois had the greatest sphingomyelin concentration in whole milk during the summer compared with the rest of the year, and compared with the other 2 Holstein herds. The greatest variation within a herd was from 636 to 1,188 $\mu\text{g/g}$ of milk fat. Although the number of farms was insufficient to reliably assess breed differences, on average, milk fat for the 3 Holstein herds sampled contained a greater concentration of sphingomyelin than did milk fat from the 2 Jersey herds (1,073 vs. 955 $\mu\text{g/g}$ of milk fat), in agreement with results from experiment 1.

CONCLUSIONS

Factors that may affect sphingomyelin concentrations in milk and milk fat were better defined in this research. Animal-specific factors, such as breed, parity, and stage of lactation, influenced sphingomyelin in milk or milk fat in some manner. Breed affected concentrations of sphingomyelin in milk fat, with Holsteins having greater concentrations than Jerseys; we speculate that this difference may be caused by differences in the fat globule size between breeds. Parity seemed to affect concentrations of sphingomyelin in milk fat, with older

cows having lower concentrations than younger cows. Stage of lactation did not affect concentrations of sphingomyelin in milk fat but did affect whole milk concentrations. Cows in late lactation had greater concentrations of sphingomyelin in whole milk, likely because of the increases in milk fat content in late lactation. Sphingomyelin concentrations in milk fat from bulk milk samples from Illinois farms were greater during the summer than during the winter. Milk fat content and globule size most likely explain the results for all of these effects. In contrast, the nutritional factors examined, intake restriction and soybean oil supplementation, did not influence the sphingomyelin concentrations in milk or milk fat. At this time, we therefore conclude that it would be both impractical and difficult for dairy producers to increase sphingomyelin concentrations by dietary modification.

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